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86081779 MEDLINE
DN 86081779 PubMed ID: 4076184
TI Purification and characterization of GDP-D-mannose 4,6-dehydratase from porcine thyroid.
AU Broschat K O; Chang S; Serif G
SO EUROPEAN JOURNAL OF BIOCHEMISTRY, (1985 Dec 2) 153 (2) 397-401. Journal code: 0107600. ISSN: 0014-2956.
CY GERMANY, WEST: Germany, Federal Republic of
DT Journal; Article; (JOURNAL ARTICLE)

Reitman ML, Trowbridge IS, Kornfeld S.
Mouse lymphoma cell lines resistant to pea lectin are defective in fucose metabolism.
J Biol Chem. 1980 Oct 25;255(20):9900-6.
PMID: 6159350 [PubMed - indexed for MEDLINE]

Two Chinese hamster ovary glycosylation mutants affected in the conversion of GDP-mannose to GDP-fucose.
AU Ripka J; Adamany A; Stanley P
NC 3PO CA13330 (NCI)
CA90173 (NCI)
R01 CA36434 (NCI)
SO ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, (1986 Sep) 249 (2) 533-45. Journal code: 0372430. ISSN: 0003-9861.
CY United States

Participation of an endogenous inhibitor of fucosyltransferase activities in the developmental regulation of intestinal fucosylation processes.
AU Ruggiero-Lopez D; Biol M C; Louisot P; Martin A
CS Department of General and Medical Biochemistry, INSERM-CNRS U. 189, France.
SO BIOCHEMICAL JOURNAL, (1991 Nov 1) 279 (Pt 3) 801-6. Journal code: 2984726R. ISSN: 0264-6021.

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Two Chinese Hamster Ovary Glycosylation Mutants Affected in the Conversion of GDP-Mannose to GDP-Fucose

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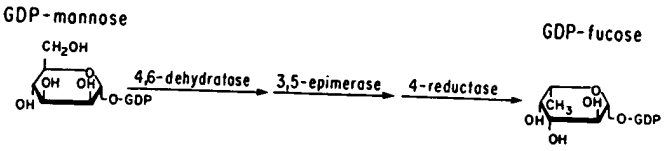
A biochemical basis for the pea and lentil lectin resistance of two Chinese hamster ovary (CHO) cell mutants, Lec13 and Lec13A, was investigated. Studies of the G glycopeptides of vesicular stomatitis virus grown in the mutants indicated that Lec13 cells essentially lack the ability to add fucose to complex carbohydrates while Lec13A cells synthesize significant proportions of fucosylated, complex moieties. However, both mutants were known to be reverted to lectin sensitivity by growth in L-fucose, making them similar to the mouse lymphoma mutant, $PL^R1.3$, which is defective in the conversion of GDP-manose to GPD-fucose [M. L. Reitman, I. S. Trowbridge, and S. Kornfeld (1980) *J. Biol. Chem.* **255**, 9900-9906]. Optimal conditions for the production of GDP-fucose from GDP-mannose by CHO cytosol were found to occur at pH 8 in the presence of 7.5 μM GDP-mannose, 15 mM Mg^{2+}, 0.2 mM NAD^+, 0.2 mM NADPH, 10 mM niacinamide, 5 mM ATP, and 50 mM Tris-HCl. Under these conditions, Lec13 cytosol produced no detectable GDP-fucose nor GDP-sugar intermediates while Lec13A cytosol produced significant quantities of both. Mixing experiments with Lec13 cytosol identified the first enzyme of the conversion pathway (GDP-mannose 4,6-dehydratase, EC 4.2.1.47) as the site of the block. In addition to being markedly reduced, the Lec13A 4,6-dehydratase activity was relatively insensitive to changes in pH in comparison to the activity in parental cytosol, suggesting that Lec13A cells might possess a structurally altered GDP-mannose 4,6-dehydratase enzyme.

Lectin-resistant (Lec^R)[2] cell lines have been useful in defining the pathways of carbohydrate biosynthesis as well as the biological roles of cellular carbohydrates. The recessive Lec^R Chinese hamster ovary (CHO) mutants characterized to date are defective in specific glycosyltransferase activities, the compartmentalization of nucleotide-sugars or in the biosynthesis of dolichyloligosaccharides [reviewed in Ref. (1)]. None of these phenotypes is reverted by growth in a simple sugar. In contrast, two recently described pea lectin-(PSA) resistant CHO mutants, Lec13 and Lec13A, were reverted to PSA sensitivity by growth in medium containing L-fucose (2). Phenotypic reversion by L-fucose was previously reported for a lymphoma line, $PL^R1.3$, that is defective in the synthesis of GDP-fucose (3). The lesion in $PL^R1.3$ cells was localized to the first enzyme in the conversion pathway of GDP-mannose to GDP-fucose. This pathway provides the major source of GDP-fucose in mammalian cells (4, 5) and has been found in bacterial plant, and animal cells (3, 6-8) to consist of the following reactions (7):

[1] Author to whom correspondence should be addressed.

[2] Abbreviations used: Lec^R, lectin-resistant; CHO, Chinese hamster ovary; PSA, pea lectin; PMB, *p*-hydroxymercuribenzoic acid; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; FCS, fetal calf serum; Con A, concanavalin A; VSV, vesicular stomatitis virus; PBS, phosphate-buffered saline; GlcNAc, *N*-acetylglucosamine; GlcNAc-T1, *N*-acetylglucosaminyltransferase.

0003-9861/86 $3.00



Alternatively, synthesis of GDP-fucose by the fucose salvage pathway (5, 9) can occur in the presence of exogenous L-fucose, allowing cells blocked in the conversion pathway to phenotypically revert (3).

The fact that both Lec13 and Lec13A CHO mutants are phenotypically reverted by L-fucose and that they belong to the same recessive complementation group (2) suggested they would both be defective in synthesizing GDP-fucose from GDP-mannose. However, the mutants display phenotypic differences: compared to parental CHO, Lec13 cells are more than 48 times resistant to PSA, while Lec13A cells are only 9 times more resistant (2). It was important, therefore, to investigate the carbohydrates synthesized by these mutants as well as their abilities to convert GDP-mannose to GDP-fucose. In this paper we show that both Lec13 and Lec13A are defective in GDP-fucose formation, although to different extents. They are both affected in the first enzyme of the conversion pathway, GDP-mannose 4,6-dehydratase (EC 4.2.1.47). Whereas Lec13 is completely deficient in this activity, Lec13A shows partial enzyme activity in cell-free extracts. However, in the intact cell, the Lec13A enzyme appears to be quite active. A preliminary report of these results has appeared (10).

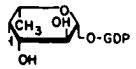
EXPERIMENTAL PROCEDURES

Materials. GDP-D-[1-^{3}H]mannose (10.7 Ci/mmol) and GDP-D-[U-^{14}C]mannose (235 mCi/mmol) were obtained from New England Nuclear; D-[6-^{3}H]-glucosamine hydrochloride (20–40 Ci/mmol), GDP-D-[U-^{14}C]mannose (228 mCi/mmol), and ACS II from Amersham Radiochemical Corporation, Arlington, Heights, Illinois; ATP, NAD^+, NADPH, niacinamide, EDTA, *p*-hydroxymercuribenzoic acid (PMB), *N*-ethylmaleimide (NEM), dithiothreitol (DTT), cetylpyridinium chloride, phenylmethylsulfonyl fluoride (PMSF), D-mannose, L-fucose, L-rhamnose, 6-deoxy-D-glucose, GDP-D-mannose, hexokinase, and Amberlite MB-3 from Sigma Chemical Company, St. Louis, Missouri; Pronase from Calbiochem-Behring, La Jolla, California; alpha medium, horse serum, and fetal calf serum (FCS) from GIBCO Laboratories, Grand Island, New York; concanavalin A- (Con A) Sepharose, from Pharmacia, Uppsala, Sweden; PSA-agarose from Vector Laboratories, Burlingame, California; Dowex 50 X-4, 200 to 400 mesh (H^+ form), Dowex 1 X-8, 200 to 400 mesh (Cl^- form), and Bio-Gel-P2 from Bio-Rad Laboratories, Richmond, California; Dowex 1 X-8, 200 to 400 mesh (formate form) was prepared from Dowex 1 X-8, 200 to 400 mesh (Cl^- form) as described by the manufacturer.

Talomethylose was purified essentially as described by Markovitz (11) from a crude polysaccharide fraction prepared from strain GS bacteria and generously provided by Dr. A. Markovitz (University of Chicago). The crude polysaccharide was dissolved in 0.04 N sodium acetate, titrated with 1% cetylpyridinium chloride until a precipitate was formed, and centrifuged at 20,000 rpm for 15 min in the SS-34 rotor of a Sorvall centrifuge. Four volumes of absolute ethanol were added to the supernatant and the mixture was placed at 4°C overnight. The sample was centrifuged at 20,000 rpm for 15 min and the pellet dissolved in 0.04 N sodium acetate. Ethanol precipitation was repeated once, and the pellet, after lyophilization, was resuspended in 1 N H_2SO_4 and boiled at 100°C for 1.5 h. The solution was diluted with 4 vol of 10% methanol, passed through a column of Dowex 50 (H^+ form) coupled to a column of Dowex 1 (formate form) (12), and eluted with 10% methanol. The effluent was dried under vacuum at 40°C, resuspended in water, and passed through an Amberlite MB-3 column. The eluate was concentrated by lyophilization, resuspended in 0.5 ml 50% ethanol, spotted on Whatman No. 1 paper and chromatographed in solvent C (see Product Identification). Alkaline silver nitrate staining of a small strip revealed a single band in the area expected for talomethylose. This band was cut out, eluted with water, and concentrated. Small aliquots were spotted on Whatman No. 1 strips and chromatographed in solvents B, D, and E (see Product Identification). Each solvent revealed a single band which chromatographed with values predicted for talomethylose (13–16).

Cell lines and cell culture. The parental CHO cell lines Pro^-5 and Gat^-2 as well as the PSA-resistant mutants Lec13 (Pro^-Lec13.6A and Gat^-Lec13.1A) and Lec13A (Pro^-Lec13A.2A) have been previously described (2). The mouse lymphoma line BW5147 and the PSA-resistant mutant $PL^R1.3$ (17) were a gift from I. S. Trowbridge (Salk Institute, San Diego). All cells

GDP-fucose



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alin A- (Con A) Sepharose, from
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esh (H^+ form), Dowex 1 X-8, 200
m), and Bio-Gel-P2 from Bio-Rad
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k Institute, San Diego). All cells

were cultured in suspension at 37°C in alpha medium containing antibiotics and 10% horse serum with 2% FCS or in 10% FCS alone. Cells were routinely screened for the presence of *Mycoplasma* by fluorescence microscopy (18) and found to be negative.

Preparation of labeled viral glycopeptides. Labeled glycopeptides were prepared from vesicular stomatitis virus (VSV, Indiana strain) grown in parental or mutant cells in the presence of [^{3}H]glucosamine as previously described (19). Virus purified by equilibrium gradient centrifugation was digested with 1 mg/ml Pronase containing 3 mM $CaCl_2$ at 50°C as described (19). After 48 h, the solution was boiled for 2 min and clarified by centrifugation.

Lectin-affinity chromatography. Pronase glycopeptides were fractionated on Con A–Sepharose (0.6 × 22 cm) and PSA-agarose (0.6 × 22 cm). Glycopeptides were applied to the columns in Con A buffer (1 M sodium chloride, 0.1 M sodium acetate, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM $MnCl_2$, 0.001 mg/ml polyethylene glycol, 0.02 mg/ml sodium azide, pH 7.3). Unretarded glycopeptides were eluted with three column volumes of Con A buffer while bound glycopeptides were eluted with Con A buffer containing 200 mM α-methylmannoside. Fractionated glycopeptides were desalted under pressure on a Bio-Gel-P2 (minus 400) column (1.5 × 46 cm).

Assay for conversion of GDP-D-mannose to GDP-L-fucose. Cells (1×10^8) growing exponentially in suspension were washed once in cold phosphate-buffered saline (pH 7.4) containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS) and centrifuged at 1200 rpm for 10 min in a GLC-2B centrifuge. The cell pellet was resuspended in cold 50 mM Tris-HCl (pH 8.0) containing 0.35 mg/ml PMSF. The cells were allowed to swell for 10 min on ice and homogenized (Dounce) 30–40 times until >95% of the cells were broken. The suspension was centrifuged at 100,000*g* for 1 h, and the supernatant (cytosol) used immediately. Cytosol could be frozen at −70°C in 50 mM Tris-HCl containing 2.5% glycerol without loss of activity for at least 2 weeks. The presence of PMSF during cell homogenization increased the yield of enzyme activity about threefold.

Optimal assay mixtures contained 600–800 μg protein, 10 μmol niacinamide, 5 μmol ATP, 0.2 μmol NAD^+, 0.2 μmol NADPH, 7.5 nmol GDP-[^{3}H]mannose (10^5 cpm), and 50 μmol Tris-HCl (pH 8.0) in a total volume of 1 ml. After varying incubation times at 37°C, the reaction was stopped by adding 50 μl of 2 N HCl, the solution was boiled in a heating block for 20 min and subsequently neutralized with 55 μl of 2 N NaOH. Quantitation of the conversion of GDP-mannose to GDP-fucose was achieved by determining the amount of fucose present after acid hydrolysis of the sugar-nucleotides. Free mannose (released from unutilized GDP-mannose) was phosphorylated by treatment with hexokinase (4 units) in the presence of 5 μmol of ATP and 5 μmol of $MgCl_2$. After 30 min at 37°C, the mixture was passed over a Dowex 1 (formate) column (0.6 × 6 cm) and eluted with water. An aliquot of the eluate was counted in ACS II in a Beckman LS9000 scintillation counter. The remainder was desalted through an Amberlite MB-3 column (0.8 × 6.2 cm), dried under vacuum at 40°C, and chromatographed in the appropriate solvent systems (see Product Identification).

Assay for production of GDP-6-deoxy-keto sugar intermediates. The assay mixture was the same as described above except GDP-mannose of higher specific activity (~10^6 cpm per reaction) was used and NADPH was omitted. After 90 min at 37°C, the solution was boiled for 10 min, and 1 mg of $NaBH_4$ was added to reduce keto intermediates. After 1.5 h at room temperature, the mixture was hydrolyzed with HCl, neutralized, treated with hexokinase, and passed through Dowex 1 (formate) as described above. The eluate was passed through a column of Amberlite MB-3 (1.3 × 10 cm), dried under vacuum, and chromatographed in the appropriate solvent systems (see Product Identification).

Product identification. Descending paper chromatography was carried out on Whatman No. 1 paper in the following solvent systems: solvent A, pyridine-ethyl acetate-water-acetic acid (5:5:3:1) with pyridine-ethyl acetate-water (2:8:1) in the bottom of the tank (16); solvent B, the upper phase of pyridine–ethyl acetate-water (1.0:3.6:1.15) (15); solvent C, 2-butanone saturated with water (13); solvent D, 2-butanone-acetic acid-saturated boric acid (9:1:1) (14); solvent E, the upper phase of butanol–ethanol–0.2 M sodium borate buffer, pH 8.94 (4:1:2) (13). When solvent E was used, the paper was impregnated with 0.2 M sodium borate, pH 8.94 and dried prior to sample application. Chromatography time varied for the different solvents: solvents A (20–24 h), B (6 h), and D (17 h) were used to identify fucose; solvents B (6 h), C (12 h), D (7 h), and E (70 h) were used to identify rhamnose and talomethylose. Sugar standards were detected by the alkaline silver nitrate procedure (20). Because the relative migrations of each sugar in these solvents vary somewhat in reports from different laboratories (13–16), the values obtained in our experiments are included in Table I. Radiolabeled compounds were detected by radioscanning of paper chromatograms by using a Packard 7201 or by cutting the paper into 1-cm strips, adding 0.5 ml water and 5 ml ACS II, and counting in a liquid scintillation spectrometer.

The purity of commercial GDP-[^{3}H]mannose was evaluated by several approaches. Over 99% of the radioactivity was bound by Dowex 1 (formate) and, after acid hydrolysis, 99% of the radiolabel passed through Dowex 1 (formate). However, following incubation with heat-inactivated cytosol, treatment with acid, neutralization by alkali, and treatment with hexokinase, a consistent level of 2–3% of the radiolabel did not bind to Dowex 1 (formate). Further treatment of this effluent with hexokinase did not reduce this value,

TABLE I

CHROMATOGRAPHIC PROPERTIES OF SUGAR STANDARDS

Sugar	Solvent				
	A	B	C	D	E
Mannose	0.78	0.45	0.34	0.43	0.35
6-Deoxygalactose (fucose)	0.88	0.67	0.57	0.66	0.28
6-Deoxyglucose (glucomethylose)	ND	0.97	0.86	ND	0.53
6-Deoxymannose (rhamnose)	1.00	1.00	1.00	1.00	1.00
6-Deoxytalose (talomethylose)	ND	1.41	2.04	1.39	0.79

Note. Descending paper chromatography in solvents A, B, C, D, and E described under Experimental Procedures was performed on Whatman No. 1 paper for various times. The mobilities of the sugars were calculated relative to rhamnose in each case (R_f = 1.00). ND, not determined.

indicating that the hexokinase step was sufficient to remove mannose (and glucose). The labeled effluent did not comigrate with any of the sugar standards tested, indicating the presence of impurities in the GDP-[^{3}H]mannose preparation. If reduction with $NaBH_4$ was performed prior to acid hydrolysis and hexokinase treatment, the impurities separated into three peaks in both solvents A and B: one peak was detected between mannose and fucose, a second peak cochromatographed with rhamnose, and a third migrated very close to talomethylose. Similar impurities were detected in GDP-[^{14}C]mannose from two sources. Because these impurities represented 2–3% of the initial radioactivity and cochromatographed in the vicinity of rhamnose and talomethylose in two solvent systems, initial experiments were interpreted to indicate that the mutant lines actually produced the GDP-6-deoxy-keto sugar intermediates expected from the action of GDP-mannose 4,6-dehydratase (10). Fortunately, the radioactive impurities did not comigrate with rhamnose and talomethylose in solvent C. Therefore the radioactive products of assays for the detection of GDP-6-deoxy-keto sugar intermediates were resolved by chromatography in solvent C after being mixed with 1 μmol each of GDP-mannose, mannose, fucose, rhamnose, and talomethylose (carrier sugars). After radioscanning, the rhamnose and talomethylose areas were cut out, eluted, and chromatographed with the mixture of carrier sugars in solvent B. Rhamnose and talomethylose regions were again cut out, eluted, and chromatographed with carrier sugars in either solvent D or solvent E. The final chromatogram was cut into 1-cm strips, 0.5 ml water and 5 ml ACS II were added, and the sample was counted by liquid scintillation. In most experiments, rhamose and talomethylose were identified by chromatography in solvent C followed by chromatography of the relevant eluted peaks in solvent D. Recovery experiments using a defined amount of [^{3}H]mannose showed an average yield of 58% after two chromatographic sequences. The data presented are not corrected for recoveries.

RESULTS

Lectin-affinity chromatography of pronase glycopeptides. The different levels of PSA resistance exhibited by Lec13 and Lec13A mutants (2) indicated that their cellular carbohydrates might also vary. To investigate this question, VSV was grown in both mutants and the structural characteristics of the N-linked complex carbohydrates associated with the G glycoprotein of the viruses were compared by lectin affinity chromatography. The major carbohydrate species of CHO/VSV is biantennary and binds to Con A–Sepharose, while the minor portion is mainly triantennary, containing a β1,6-linked *N*-acetylglucosamine (GlcNAc) residue, and does not bind to Con A–Sepharose (21). Both bi- and triantennary moieties contain α1,6-linked fucose on ~30% of their molecules (21), conferring the ability to bind to PSA-agarose (22). The abilities of the mutants to fucosylate N-linked carbohydrates was assessed by preparing Pronase glycopeptides from Lec13/VSV and Lec13A/VSV and comparing their behavior during chromatography on Con A–Sepharose and PSA-agarose.

The proportion of biantennary glycopeptides from Lec13/VSV, Lec13A/VSV, and parental CHO/VSV were determined

IDARDS

ent

	D	E
4	0.43	0.35
7	0.66	0.28
6	ND	0.53
0	1.00	1.00
4	1.39	0.79

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RESULTS

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Lec13/VSV, Lec13A/VSV,
HO/VSV were determined

by Con A-Sepharose chromatography to be 70-80% in each case (data not shown). These glycopeptides and those that passed through Con A-Sepharose (branched) were fractionated on PSA-agarose (Fig. 1). In both cases, Lec13/VSV contained <1% glycopeptides that bound to PSA-agarose (i.e., <1% contained an α1,6-linked fucose residue). In contrast, Lec13A/VSV glycopeptides contained a substantial proportion of fucosylated molecules—similar to parental CHO/VSV for biantennary moieties but reduced by ~50% in the case of branched carbohydrates. This result revealed another substantial phenotypic difference between Lec13 and Lec13A cells.

In vitro conversion of GDP-mannose to GDP-fucose. As mentioned previously, the ability of L-fucose to phenotypically revert Lec13 and Lec13A mutants suggested that their respective enzyme lesions might be in the GDP-mannose to GDP-fucose pathway. The *in vitro* assay developed by others (3, 7, 8) relied on the ability to separate and identify mannose and fucose in acid hydrolysates of the incubation mixtures

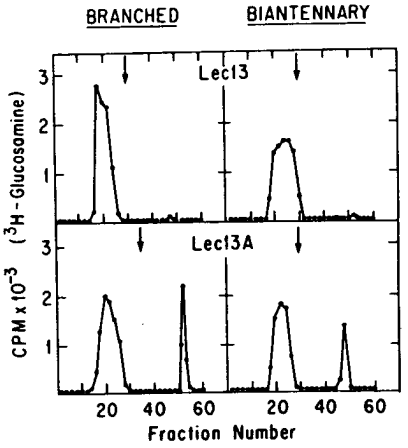


FIG. 1. Lectin affinity chromatography of viral glycopeptides. VSV glycopeptides separated by Con A-Sepharose chromatography into branched (not bound to Con A) and biantennary (Con A-bound) fractions were chromatographed on PSA-agarose. The glycopeptides that passed through PSA eluted with the void volume (V_0) while PSA-bound glycopeptides eluted after application of 200 mM α-methylmannoside (arrow). Apparent differences in V_0 are due to variation in applied sample volume.

and thus estimate the conversion process. Under conditions known to give good activity for BW5147 extracts (3), CHO cytosol produced only 3 pmol GDP-fucose/mg protein/min (Fig. 2) compared to 28 pmol GDP-fucose/mg protein/min produced by BW5147 cytosol. The latter result is in good agreement with previous values reported for BW5147 (3). The assay for CHO cytosol was therefore optimized by varying the reaction conditions and introducing a hexokinase step after acid hydrolysis (see Experimental Procedures). The latter treatment completely removed unconverted substrate from the hydrolysate (Fig. 2) and allowed the clear identification of fucose (Fig. 2) as well as critical intermediates (see below).

Optimization of the conversion activity of CHO extracts. Subcellular fractionation of CHO homogenates indicated that virtually all enzyme activity was recovered in the cytosol fraction. No activity was found in the particulate fraction. Detergent extracts (2.5% Triton X-100 or 0.75% NP-40) were reduced in activity by ~30% compared to a 100,000*g* cytosol. The 100,000*g* cytosol, prepared in the presence of PMSF, gave the best activity.

To investigate optimal conditions for the conversion activity of CHO cytosol, the effects of varying GDP-mannose concentrations (Fig. 3), divalent cation concentrations (Fig. 4), and pH (Fig. 5) were examined. Cofactor concentrations were maintained at the levels used previously by Reitman *et al.* (3). The production of GDP-fucose was maximal at 6–9 μM GDP-mannose both in the presence and absence (Fig. 3) of divalent cations. All three divalent cations tested (Ca^{2+}, Mn^{2+}, and Mg^{2+}) stimulated the production of GDP-fucose about twofold (Fig. 4). The pH optimum of the conversion activity was pH 8, both in the presence and absence of Mg^{2+} (Fig. 5). At pH 8 in the presence of 7.5 μM GDP-mannose and 15 mM Mg^{2+}, the conversion activity was linear for protein concentrations of 100–1000 μg/ml (Fig. 6). Below 100 μg/ml, linearity was reduced, as observed previously with lymphoma cell extracts (3). After a lag period of 10–20 min, the conversion activity of CHO cytosol

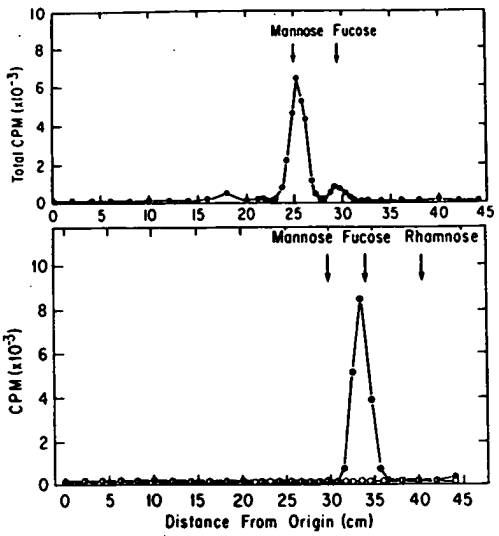


FIG. 2. Conversion of GDP-mannose to GDP-fucose by parental CHO cells. Pro⁻5 cytosol (515 μg protein) was incubated with 10 mM niacinamide, 5 mM ATP, 0.2 mM NAD^+, 0.2 mM NADPH, 3 μM GDP-[^{3}H]mannose (36,000 cpm), and 50 mM Tris-HCl (pH 8) for 60 min at 37°C. The reaction mixture was acid hydrolyzed, desalted on Amberlite MB-3, and chromatographed in solvent A for 24 h (upper panel). Pro⁻5 cytosol (●; 800 μg protein) or boiled Pro⁻5 extract (○) were incubated under optimum assay conditions including 15 mM Mg^{2+}. After 90 min at 37°C, the reaction mixture was acid hydrolyzed, treated with hexokinase, and passed through Dowex 1 (formate). The eluate was desalted on Amberlite MB-3 and chromatographed in solvent A for 24 h (lower panel). Sugar markers from parallel strips were identified by alkaline silver nitrate staining in each experiment.

was proportional to the time of incubation at 37°C (Fig. 7). Thus optimal conditions for the *in vitro* conversion of GDP-mannose to GDP-fucose by CHO cytosol occurred in

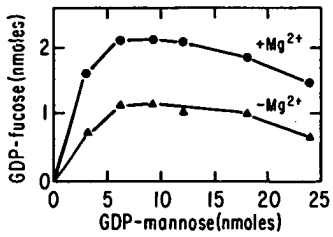


FIG. 3. Effect of substrate concentration on conversion. Pro⁻5 CHO extracts were incubated under optimum assay conditions in the presence (●) or absence (▲) of 15 mM Mg^{2+}.

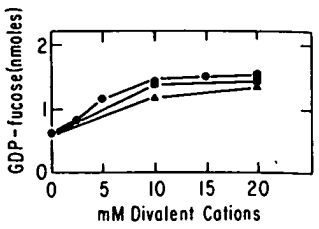


FIG. 4. Effect of divalent cations on conversion. Pro⁻5 extracts were incubated for 90 min under optimum assay conditions in increasing concentrations of Mg^{2+} (●), Mn^{2+} (■), or Ca^{2+} (▲).

the presence of 600–800 μg cytosol protein. 10 mM niacinamide, 5 mM ATP, 0.2 mM NAD^+, 0.2 mM NADPH, 7.5 μM GDP-mannose, 15 mM Mg^{2+}, and 50 mM Tris-HCl (pH 8). Incubations were usually carried out for 90 min at 37°C.

In the absence of NADPH and Mg^{2+}, conversion of GDP-mannose to GDP-fucose was not detected. However, in reactions lacking NADPH and containing 15 mM Mg^{2+} or 15 mM Ca^{2+}, production of GDP-fucose was observed. The conversion activity obtained with 15 mM Mg^{2+} alone was similar to that obtained with NADPH and 15 mM Mg^{2+} together, while in the presence of 15 mM Ca^{2+}, the conversion activity was similar to that obtained with NADPH alone. The biochemical basis of these complex ionic effects is unknown.

Pro⁻5 CHO produced 13.8 pmol GDP-fucose/mg protein/min in the absence of Mg^{2+} and another parental CHO line (Gat⁻2) showed slightly higher activity

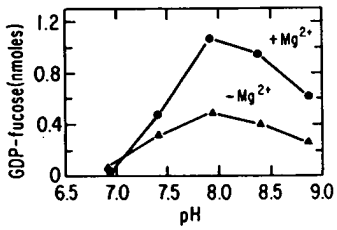
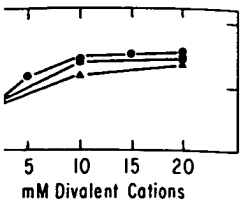


FIG. 5. pH optimum for conversion. Pro⁻5 cells were homogenized (Dounce) in low salt (5 mM Tris-HCl, pH 8) and centrifuged at 100,000*g* for 1 h. The pH was adjusted with 0.2 M Tris-HCl (pH 7-9) to a final concentration of 50 mM Tris-HCl. Reaction mixtures were incubated for 90 min under optimal assay conditions in the presence (●) or absence (▲) of 15 mM Mg^{2+}.



of divalent cations on conversion. re incubated for 90 min under optitions in increasing concentrations (■), or Ca^{2+} (▲).

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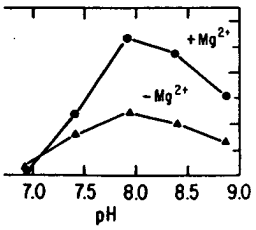

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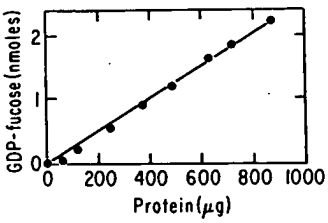

FIG. 6. Effect of protein concentration on conversion. Increasing amounts of Pro⁻5 extract were incubated for 90 min under optimum assay conditions in the presence of 15 mM Mg^{2+}.

(Table II). The activities of both CHO extracts were increased more than twofold by 15 mM Mg^{2+} and became comparable to that of other mammalian cells or tissues (3, 7). Under the conditions found to be optimal for CHO cytosol, the conversion activity of BW5147 cytosol was also improved (80 pmol GDP-fucose/mg protein/min was produced in the absence of Mg^{2+}, while in its presence, 117 pmol GDP-fucose/mg protein/min was obtained).

Inhibition of conversion activity. Concentrations of GDP-mannose above 12 μM inhibited the conversion activity of CHO cytosol (Fig. 3). This effect was probably mediated by the nucleotide since GDP-glucose at 12 μM also inhibited GDP-fucose formation (data not shown) as observed previously for plant cell extracts (8). Although divalent cations stimulated conversion activity (Fig. 4), the inclusion of 10 mM EDTA did not inhibit the activity of CHO cytosol to which no divalent cations had been added. Therefore divalent cations were not absolutely required for conversion activity. In contrast, the inhibitory effects of several sulfhydryl-blocking agents indicated the requirement of a free sulfhydryl group for conversion activity. Inclusion of 1 mM PMB reduced the conversion activity of CHO cytosol to <1% of normal. Other agents, such as NEM (10 mM) and oxidized glutathione (2 mM), also inhibited GDP-fucose production by >99%. However, 10 mM DTT had no inhibitory effect. The combined data suggest that an essential sulfhydryl group is required for the conversion of GDP-mannose to GDP-fucose by CHO cytosol.

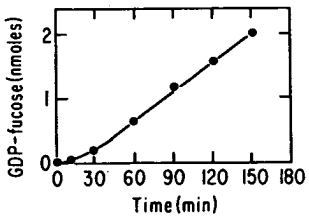

FIG. 7. Effect of time on conversion. Pro⁻5 extracts were incubated for various times under optimum assay conditions in the presence of 15 mM Mg^{2+}.

TABLE II

CONVERSION OF GDP-MANNOSE TO GDP-FUCOSE BY PARENTAL AND LEC^R CHO CYTOSOLS

Cell extract	Mg^{2+} (15 mM)	Specific activity (pmol GDP-fucose/mg protein/min)	Relative activity (%)
Boiled	–	0.1 (2)[a]	0.8
	+	0.1 (2)[a]	0.8
Parent			
Pro⁻5	–	13.80 ± 5.35 (29)	100.0
	+	30.52 ± 9.21 (26)	221.2
Gat⁻2	–	15.56 ± 2.24 (8)	112.8
	+	36.31 ± 3.18 (9)	263.1
Lec13			
Pro⁻Lec13.6A	–	0.06 ± 0.016 (5)[a]	0.44
	+	0.06 ± 0.02 (5)[a]	0.44
Gat⁻Lec13.1A	–	0.1 (2)	0.8
Lec13A			
Pro⁻Lec13A.2A	–	0.45 ± 0.11 (3)[a]	3.3
	+	0.47 ± 0.10 (3)[a]	3.4

Note. Cell extracts were incubated under optimum assay conditions with 10^5 cpm GDP-[^{3}H]mannose in the presence or absence of 15 mM Mg^{2+}. GDP-fucose production was calculated as the total counts per minute eluting through Dowex 1 (formate) corrected for background determined with a boiled extract control. Specific activities with standard deviation based on the number of observations (in parentheses) are given.

[a] These samples were incubated under optimal conditions containing 10^6 cpm GDP-[^{3}H]mannose in the presence or absence of 15 mM Mg^{2+}. GDP-fucose formation was determined by paper chromatography of the products that eluted through Dowex 1 (formate).

Conversion activities of mutant cells. Two independent Lec13 mutants gave no detectable GDP-fucose in the presence or absence of Mg^{2+} (Table II). Chromatograms of Lec13 products were coincident with those of boiled reaction mixtures (Fig. 8). Similar results were obtained with the

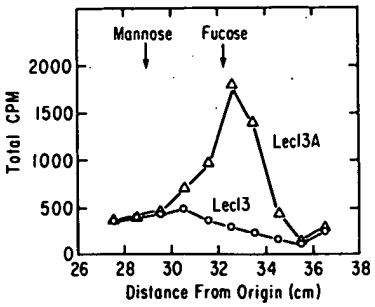

FIG. 8. Conversion by Lec13 and Lec13A cytosols. Pro⁻Lec13 (O) and Pro⁻Lec13A (Δ) cytosols were incubated for 90 min under optimum assay conditions with 7.5 μM GDP-[^{3}H]mannose (~10^6 cpm per reaction) in the absence of Mg^{2+}. The products were chromatographed in solvent C for 12 h. The Lec13 products appeared identical to those obtained with a boiled cytosol.

BW5147 mutant $PL^R1.3$, which exhibited <0.1% of parental BW5147 conversion activity. In contrast, the Lec13A CHO mutant possessed approximately 3% of parental CHO conversion activity (Fig. 8), although this activity was not stimulated by Mg^{2+} under the usual assay conditions (Table II). If, however, Lec13A cytosol was prepared in 5 mM Tris-HCl (pH 8) instead of the usual 50 mM Tris-HCl (pH 8) buffer, the production of GDP-fucose was stimulated twofold by Mg^{2+}, as observed with parental CHO (data not shown).

Parental CHO and mutant cytosols were mixed to determine if a soluble inhibitor of GDP-mannose to GDP-fucose conversion activity was present. When equal amounts of Pro⁻5 and Pro⁻Lec13 or Gat⁻2 and Gat⁻Lec13 cytosols were mixed, the expected activity for parental extract alone was obtained. In addition these mixtures showed stimulation of GDP-fucose production by Mg^{2+}. Mixing Pro⁻5 and Pro⁻Lec13A extracts in the absence or presence of Mg^{2+} also resulted in the activity expected for the parental extract. Therefore, neither mutant type appears to contain an inhibitor of GDP-mannose conversion activity.

In vitro production of nucleotide-sugar intermediates. The conversion of GDP-mannose to GDP-fucose occurs via GDP-6-deoxy-keto sugar intermediates in bacteria (7) and appears to be similar in lymphoma cells (3). In the absence of NADPH, GDP-6-deoxy-keto sugar intermediates are therefore expected to accumulate (see Fig. 9). The intermediates may be reduced with $NaBH_4$, hydrolyzed to yield 6-deoxy sugars, and characterized by paper chromatography. In contrast to mannose, the reduced intermediates should not be phosphory-

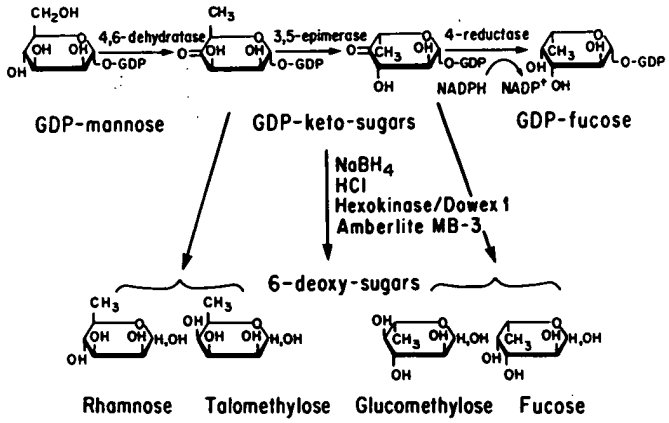

FIG. 9. Assay for nucleotide-sugar intermediates. The proposed conversion pathway of GDP-mannose to GDP-fucose (7). Assay mixtures lacking exogenous NADPH accumulate GDP-keto sugar intermediates which, after reduction with NaBH4, acid hydrolysis, treatment with hexokinase/Dowex 1, and desalting on Amberlite MB-3, may be detected as 6-deoxy sugars by paper chromatography.

lated by hexokinase and could therefore be separated from mannose by the hexokinase step, making the assay for intermediates quite sensitive. It was expected that, following $NaBH_4$ treatment of the assay mixtures, rhamnose and talomethylose would be derived from the first intermediate and 6-deoxyglucose and fucose from the second intermediate (7). In fact, reduced extracts of parental CHO cells yielded only rhamnose and talomethylose (Fig. 10). The products predicted to arise from the second intermediate were not detected, as reported previously by others (3, 7).

The products of the assay for intermediates were first chromatographed in solvent C; bands comigrating with rhamnose and talomethylose were eluted and subsequently chromatographed separately in solvents B, D, and E. The radiolabeled products comigrated with authentic rhamnose and talomethylose standards in each solvent system (see Table I). The ratio of rhamnose to talomethylose was approximately 1:2–2.5 and represented a conversion of 1–1.5% of the GDP-mannose in the reaction mixture. Although, as mentioned previously, 1 mM PMB blocked the overall conversion of GDP-mannose to GDP-fucose by parental CHO cytosol, under similar conditions both rhamnose and talomethylose were produced at 60–75% of parental levels (data not shown). This suggests that the essential sulfhydryl required for GDP-fucose production is not associated with the GDP-mannose 4,6-dehydratase activity. However, increasing the concentration of PMB to 10 mM inhibited the production of 6-deoxy sugars completely. This may reflect a requirement for NAD^+ by the first enzyme activity as observed by others (8, 23, 24).

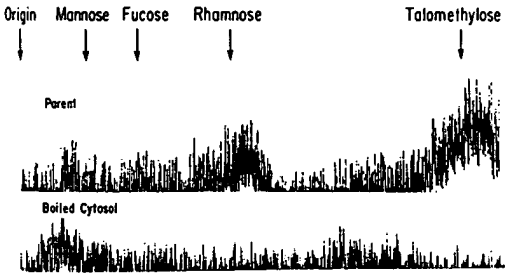

FIG. 10. Conversion of GDP-mannose to GDP-sugar intermediates. Pro⁻5 cytosol or boiled cytosol was incubated under optimum assay conditions in the absence of Mg^{2+} and NADPH. The products were chromatographed in the presence of carrier sugars in solvent C for 12 h and radioscanned. Authentic sugar standards were chromatographed in parallel.

The production of 6-deoxy sugars was approximately linear up to 90 min of incubation at 37°C. However, the overall conversion of GDP-mannose to intermediates was only 1–1.5% of the GDP-mannose present in the reaction mix compared to the 15–30% conversion of GDP-mannose to GDP-fucose obtained under optimal assay conditions (Table II). The low levels of intermediates formed may reflect the instability of the GDP-6-deoxy-keto sugars (7) or some type of feedback inhibition.

Lec13 cells produced no detectable rhamnose in incubation mixtures lacking exogenous NADPH (Table III). The small number of counts per minute from Lec13 cytosol that comigrated with talomethylose represented <5% of the counts obtained from parental cytosol. It seems likely that these counts did not represent authentic talomethylose since, if they did, ~500 cpm would be expected to have comigrated with rhamnose. In two experiments, no counts above the background level were detected in the rhamnose area. On the other hand, Lec13A cells produced both rhamnose and talomethylose to approximately 11% of parental levels (Table III).

If the mutations expressed by Lec13 and Lec13A cells affect the first enzyme, as reported for the $PL^R1.3$ mutant (3), Lec13 cytosol should be able to convert GDP-6-deoxy-keto sugar intermediates (provided from another source) to GDP-fucose. To generate GDP-sugar intermediates, parental cytosol was incubated in the absence of NADPH for 60 min, boiled to inactivate enzyme activities, and fresh parental CHO or Lec13 cell cytosols were added to compare their abilities to complete the pathway. Both parental CHO and Lec13 cytosols produced GDP-fucose in the presence of exogenous NADPH (Table IV). In fact, Lec13 was able to produce GDP-fucose as well as parental extracts under these conditions, providing evidence that the en-

TABLE III

GDP-KETO SUGAR INTERMEDIATES PRODUCED BY PARENTAL AND MUTANT CYTOSOLS

Cell extract	Rhamnose (total cpm)	Talomethylose (total cpm)	Specific activity (pmol 6-deoxyhexose/ mg protein/min)
Boiled	150	180	0.019
Pro⁻5	8620	22,400	1.30
Pro⁻Lec13.6A	160	1,160	0.046
Pro⁻Lec13A.2A	1500	3,700	0.14

Note. Parental and mutant cytosols were incubated under optimum assay conditions with 3×10^6 cpm GDP-[^{3}H]mannose per tube, in the absence of NADPH and Mg^{2+}. The products were chromatographed in the presence of carrier sugars in solvent C for 12 h and radioscanned. Rhamnose and talomethylose areas were cut out, eluted, and chromatographed in solvent D for 7 h. The rhamnose and talomethylose areas were again cut out, eluted, and counted.

zyme activities subsequent to the GDP-mannose 4,6-dehydratase activity are unaffected by the Lec13 mutation. Since Lec13A cells belong to the same complementation group (2), the Lec13A mutation must also affect the GDP-mannose 4,6-dehydratase activity.

GDP-mannose 4,6-dehydratase pH optimum. If the Lec13A mutation reduces but does not abolish GDP-mannose 4,6-dehydratase activity, this enzyme might exhibit properties different from those of the same enzyme in parental CHO. The pH optimum of the GDP-mannose 4,6-dehydratase was therefore examined in parental and Lec13A extracts. Parental CHO showed a sharp optimum of pH 7.5 and little activity at pH 7 (Fig. 11). Lec13A extracts also showed optimum activity at pH 7.5. However, the Lec13A extracts exhibited only slightly lower activity at pH 7. This contrasts dramatically with the activity of parental CHO cytosol which was 10 times lower at pH 7 than at pH 7.5. Thus the Lec13A 4,6-dehydratase activity appears less sensitive

TABLE IV

CONVERSION OF GDP-KETO SUGAR INTERMEDIATES TO GDP-FUCOSE

Cell extract	NADPH	Total cpm	Specific activity (pmol GDP-fucose/mg protein/min)
Boiled	+	630	0.14
Pro⁻5	−	850	0.21
	+	14,760	3.75
Pro⁻Lec13.6A	−	860	0.21
	+	14,100	3.63

Note. Pro⁻5 extracts were incubated under optimum assay conditions containing 10^6 cpm GDP-[^{3}H]mannose per tube, in the absence of NADPH and Mg^{2+} for 60 min and boiled for 10 min. Fresh Pro⁻5 or Pro⁻Lec13 cytosol was added and the incubation reinitiated in the presence or absence of NADPH for 60 min. The products were chromatographed in solvent D for 17 h, cut out, eluted, and counted.

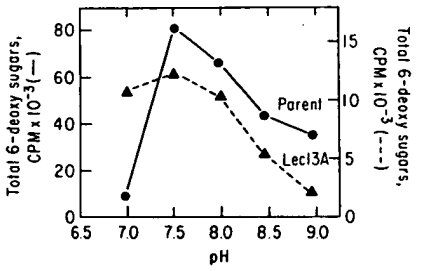

FIG. 11. pH optimum of GDP-mannose 4,6-dehydratase. Pro⁻5 (●) and Pro⁻Lec13A (▲) cytosols prepared as described in Fig. 5 were incubated for 90 min at different pHs under optimum assay conditions with 3×10^6 cpm GDP-mannose per tube in the absence of NADPH and Mg^{2+}. The products were chromatographed in the presence of carrier sugars in solvent C for 12 h. The total GDP-6-deoxy sugars produced represent the sum of rhamnose and talomethylose eluted from the chromatograms in each case (10,000 cpm = 1 pmol of 6-deoxy sugars).

MUTANT CYTOSOLS

Specific activity (pmol 6-deoxyhexose/ mg protein/min)
0.019
1.30
0.046
0.14

nditions with 3×10^6 cpm GDP-
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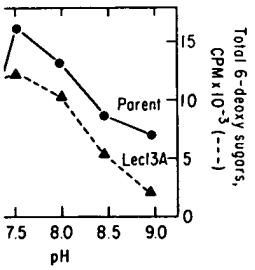

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DISCUSSION

The biosynthetic conversion of GDP-mannose to GDP-fucose occurs in bacterial, plant, and mammalian tissues (3, 6–8). The pathway is thought to require three enzyme activities [(7); see Fig. 9] that are common to a variety of other sugar–nucleotide conversions (26). In this paper, optimum conditions for the conversion of GDP-mannose to GDP-fucose by CHO cells were determined. An optimum GDP-mannose substrate concentration of approximately 7.5 μM and a pH optimum of 8 for the overall pathway was obtained, as observed previously for bacterial extracts (27, 28). In both CHO and BW5147 cell extracts, conversion activity was stimulated twofold by divalent cations. This finding has not been previously reported for mammalian cells. Indeed in plants, divalent cations are thought to inhibit the conversion (8). Interestingly, the inclusion of 15 mM Mg^{2+} replaced the requirement for NADPH, by some unknown mechanism, perhaps implicating a possible requirement for a sulfhydryl group in the pathway. The inhibition of GDP-fucose production by PMB and other sulfhydryl-blocking reagents was consistent with this hypothesis.

Under optimum conditions, Lec13 cytosol produced neither GDP-fucose nor the first nucleotide-sugar intermediate of the conversion pathway. However, Lec13 extracts were able to produce GDP-fucose when GDP-6-deoxy-keto sugar intermediates were provided by parental extracts. Thus the Lec13 mutation affects the first reaction of the conversion pathway, making the Lec13 mutant phenotypically identical to the PSA-resistant BW5147 mutant termed $PL^R1.3$ (3). Attempts to establish genetic identity between the mutants were not successful because the intraspecific hybrids necessary to perform complementation analysis were not readily obtained (J. Ripka, unpublished observations). Consistent with an inability to synthesize GDP-fucose, the carbohydrates of Lec13/VSV essentially lacked fucose residues. This correlates with the high PSA resistance of Lec13 cells since PSA requires an α1,6-linked fucose residue attached to the Asn-GlcNAc of complex carbohydrates for high-affinity binding (22).

Lec13A is a new mutant that is also affected in GDP-mannose 4,6-dehydratase activity. In Lec13A cells, however, the enzyme possesses partial activity and exhibits an altered pH sensitivity. Though a pH of 7.5 was optimal for the GDP-mannose 4,6-dehydratase of both parental and Lec13A cytosols, parental cytosol was comparatively inactive at pH 7 whereas Lec13A activity was only slightly decreased at this pH. The fact that, under the usual assay conditions, Lec13A cytosol was not stimulated by Mg^{2+} to produce more GDP-fucose is also different from the result obtained with parental cytosol. However, it could not be determined whether this affect was localized to the 4,6-dehydratase since, in the absence of NADPH and the presence of Mg^{2+}, no intermediates were detectable because GDP-fucose was produced. Lec13A cytosol prepared under low-salt conditions (5 mM instead of 50 mM) was, in fact, stimulated by Mg^{2+}, suggesting that perhaps one of the affects of Mg^{2+} is on the first enzyme since parental cytosol behaves similarly under both salt conditions.

Interestingly, Lec13A cytosol possessed 11% of parental CHO GDP-mannose 4,6-dehydratase activity, although it had only 3% of parental activity for the overall conversion of GDP-mannose to GDP-fucose. This suggests that the GDP-mannose 4,6-dehydratase enzyme may not be the rate-limiting step in the *in vitro* pathway. Consistent with this, the pH optimum for the overall pathway is 8 whereas the optimum for the 4,6-dehydratase reaction is 7.5. It is also possible that the Lec13A mutation affects more than one enzyme of the conversion pathway. This may occur indirectly if the enzymes of the pathway act in a complex which is disrupted by the altered Lec13A GDP-mannose 4,6-dehydratase. Such a possibility would be consistent with the fact that, similar to Lec13 cells, hybrids of Lec13 and Lec13A are highly PSA-resistant (2), whereas the Lec13A phenotype (in providing partial 4,6-dehydratase ac-

tivity) might be expected to behave dominantly in such hybrids. However, the Lec13A mutant was derived by single step selection from an unmutagenized culture (2), making it unlikely that it would be the result of more than one mutation. In addition, Lec13A cells clearly synthesize substantial amounts of GDP-fucose in intact cells since Lec13A/VSV carbohydrates are highly fucosylated (Fig. 1). Therefore the pathway subsequent to the first reaction appears to be intact. The combined data suggest that the Lec13A mutation reduces GDP-mannose 4,6-dehydratase activity directly by altering its structure, presumably by mutation of the gene that codes for the enzyme.

Several glycosylation defects that give intermediate phenotypes apparently due to partial activity of the affected enzyme or transport activity have now been isolated (29). The best characterized of these is Lec1A, a mutant that exhibits partial UDP-GlcNAc:α-D-mannoside (GlcNac β1,2 to Man α1,3) *N*-acetylglucosaminyltransferase (GlcNAc-T1) activity. The kinetic and biochemical properties of the Lec1A enzyme are different from parental GlcNAc-T1 in cell-free extracts (30). However, under appropriate assay conditions the Lec1A enzyme exhibits a V_{max} equivalent to the parental enzyme, providing good evidence that the Lec1A mutation affects the structural gene for GlcNAc-T1.[3] The defective GlcNAc-T1 of Lec1A mutants is quite active in the intact cell (30), as appears also to be the case for the Lec13A 4,6-dehydratase. It is apparent from Fig. 1 that glycopeptides of Lec13A/VSV were almost as fucosylated as those of CHO/VSV. This finding correlates with the relative PSA sensitivity of Lec13A cells. They are 5 times more sensitive to PSA than Lec13 mutants (2).

Clearly much remains to be learned of the enzymes and cofactors required for the conversion of GDP-mannose to GDP-fucose in animal cells. Different mutations affecting the pathway should continue to define it. Unfortunately it is not easy to perform or interpret kinetic experiments for such a complex set of reactions occurring in a crude cytosolic extract. Thus, the evidence that the Lec13A 4,6-dehydratase possesses a structural defect is necessarily indirect. However, an understanding of carbohydrate biosynthesis at the molecular level should eventually be possible when the genes that code for glycosylation enzymes are cloned and mutations that affect their activity are identified. Both the Lec13 and Lec13A mutants, since they belong to the same complementation group, would be expected to carry a structural mutation in the gene that codes for GDP-mannose 4,6-dehydratase. One of the mutations (Lec13) appears to inactivate enzyme activity while the other (Lec13A) gives reduced enzyme activity. Both mutants should ultimately provide insight into the structure/function relationships of this enzyme.

[3] W. Chaney and P. Stanley, *J. Biol. Chem.*, in press.

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